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## **NLRP3 inflammasome as a key player in pericarditis: rationale for a targeted therapeutic strategy**

### **Relatore**

Chiar.mo Prof. Fabrizio Montecucco

### **Candidato**

Dott. Aldo Bonaventura

### **Correlatore**

Chiar.mo Prof. Antonio Abbate

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*The herein reported PhD research project has reached its full development during the last two years and has been published in the form of an original research article in a peer-reviewed scientific journal.*

*As such, this PhD thesis almost completely reproduces the relevant publication that can be found in JACC Basic Transl Sci. 2021 Feb 22;6(2):137-150. doi: 10.1016/j.jacbts.2020.11.016. eCollection 2021 Feb.*

## SUMMARY

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## ***INTRODUCTION***

Acute pericarditis is a clinical syndrome characterized by an intense inflammatory response involving the pericardium, generally occurring in healthy individuals as an isolated entity or as a manifestation of a systemic disease (1). Acute pericarditis was reported in 27.7 cases per 100,000 person-years in an Italian urban area, being responsible for 0.1% of all hospital admissions and 5% of emergency room admissions for chest pain (2).

Although its pathogenesis is not completely understood (3), it has been hypothesized that acute pericarditis represents a stereotypical response to an acute injury to the mesothelial cells of the pericardium (4). The trigger, an 'irritant', such a virus or cellular debris following a viral infection, may activate the NACHT, leucine-rich repeat, and pyrin domain-containing protein 3 (NLRP3) inflammasome, a macromolecular intracellular complex evolved to sense stress or injury and trigger a local or systemic inflammatory response through the release of pro-inflammatory cytokines, such as interleukin (IL)-1 $\beta$  (5,6). The NLRP3 inflammasome is formed by the oligomerization of: (i) a sensor, namely NLRP3, which is a member of the nucleotide-binding oligomerization domain-containing protein (NOD)-like receptor (NLR) proteins; (ii) a scaffold protein, the apoptosis-associated speck-like protein containing a COOH-terminus caspase activation and recruitment domain (ASC); and (iii) an effector, caspase-1, whose activation is strictly required for the activity of the inflammasome as it cleaves pro-IL-1 $\beta$  to the active IL-1 $\beta$  (6). IL-1 $\alpha$  is another isoform of the IL-1 family that is released during cellular injury, functioning as an alarmin, and triggering the same



inflammatory signaling than IL-1 $\beta$  (7,8). Given the limited knowledge on its pathophysiology, there are limited treatment options for acute pericarditis (9), mostly consisting of non-steroidal anti-inflammatory drugs (NSAIDs) and colchicine, with the latter recently being recognized as an NLRP3 inflammasome inhibitor and very effective both in acute and recurrent pericarditis (10). Recent data also suggest beneficial effects of IL-1 blockade with anakinra (4,11). However, none of these treatments has been approved by the US Food and Drug Administration (FDA) for the treatment of pericarditis in humans. The clinical effect of these drugs is hypothesized to be an indirect evidence of the involvement of the NLRP3 inflammasome signaling in the pathophysiology of pericarditis. However, to date no direct demonstration of the presence of the NLRP3 inflammasome in the pericardium during an acute episode of pericarditis has been provided. The dearth of pathology studies in patients and of animal models of acute pericarditis are likely to explain the limited understanding of this syndrome and the lack of targeted therapies, which are urgently needed to reduce the disease morbidity.

The current study aimed i) to detect the presence of the NLRP3 inflammasome in human pericarditis samples, ii) to develop an innovative mouse model of acute pericarditis secondary to NLRP3 inflammasome activation, and (iii) to evaluate whether an inhibitory strategy against the NLRP3 inflammasome itself or cytokines involved in the activity of the inflammasome, i.e. IL-1 $\beta$  as well as IL-1 $\alpha$ , could be beneficial as a future treatment in patients with pericarditis.

## **MATERIAL AND METHODS**

### *Human pericardial samples*

The Pathology Service of the University of Trieste (Italy) provided human samples of patients with chronic pericarditis experiencing an acute flare and showing a constrictive evolution who needed pericardiectomy (n=6, cases) and pericardial samples from autopsies of patients who died for causes not related to pericardial syndromes (n=5, controls). The study was approved by the Ethical Committee of the Azienda Sanitaria-Universitaria Integrata Trieste (ASUITS), Trieste, Italy. Patients gave their consent before entering the study.

### *Experimental mouse model of acute pericarditis*

Adult male outbred CD1 (8–12 weeks of age) mice were supplied by Envigo (Frederick, MD, USA). The experiments were conducted under the Guidelines of Laboratory Animals for Biomedical Research published by the National Institutes of Health (No. 85-23, revised 2011). The study protocol was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Prior to surgery, mice were put under general anesthesia using intraperitoneal injection of pentobarbital sodium (50-70 mg/kg) and shaved on the lateral left side of the thorax. Mice were then orotracheally intubated and ventilated using a rodent ventilator (Minivent, Harvard Apparatus, Holliston, MA, USA). The heart was accessed with the animal lying recumbent in the right lateral decubitus position by making a ventral to dorsal incision following the curve of the rib cage approximately in the region of the 3<sup>rd</sup> and 4<sup>th</sup> rib (**Figure 1**). Muscle layers were then bluntly

dissected to expose the interosseous space between the 3<sup>rd</sup> and 4<sup>th</sup> rib granting access to the thoracic cavity. Finally, 1 mg of zymosan A (Sigma Aldrich, St. Louis, MO, USA) dissolved in 50 µl of sterile NaCl 0.9% was injected under direct visualization into the pericardial space by carefully lifting the pericardial sac with curved-tip forceps, using a 30 G needle, until a complete distribution of the solution into the pericardium was observed (**Figure 1**). The muscle layers and skin were then brought together and closed with 5.0 sutures. Zymosan A is a cell wall extract from the yeast *Saccharomyces cerevisiae* and a known agonist of the toll-like receptor-2 (TLR-2). Zymosan A leads to the activation of the NLRP3 inflammasome (12) and was already used in mouse models of peritonitis and arthritis (13,14). Zymosan A dose was chosen based on previous experiments conducted by our group on a model of peritonitis induced by the intraperitoneal injection of zymosan A (1 mg/mouse) (15).

Sham procedures were performed, where mice underwent the same surgical procedure injecting an equal volume of sterile NaCl 0.9% in the pericardial space (n=4-6).

#### *Treatment of mice following induction of acute pericarditis*

Following the recovery after surgery, mice (6-8 per group) were randomly allocated to one of 6 different treatments performed by intraperitoneal injection (i.p.) over a period of 1 week: 1) ibuprofen (100 mg/kg/day, Sigma-Aldrich, St. Louis, MO, USA); 2) colchicine (100 µg/kg/day, Enzo Life Sciences, Farmingdale, NY, USA); 3) an NLRP3 inflammasome inhibitor (NLRP3inh) (16673-34-0, 100 mg/kg/day) (16-18); 4) anakinra, recombinant human IL-1 receptor antagonist (100

mg/kg/bid, Swedish Orphan Biovitrum, Stockholm, Sweden); 5) recombinant murine IL-1 trap, (1, 5 and 30 mg/kg/day every 48h, Regeneron Pharmaceuticals, Tarrytown, NY, USA), a fusion protein mouse homologue of rilonacept, which combines the ectodomains of the IL-1 type 1 receptor (IL-1R1) and IL-1 receptor accessory protein (IL-1RAcP) to bind and block IL-1 $\alpha$  and IL-1 $\beta$ , as previously tested by our group (19); 6) matching volume of vehicle (NaCl 0.9%). All drugs were administered after surgery and then once daily with the exception of anakinra, given twice daily, and IL-1 trap, given once every 48 hours.

All tested doses of anakinra, NLRP3inh and ibuprofen were determined after a thorough review of the previous literature and validated in our laboratory (16,17,20-22). IL-1 trap was administered at 3 different doses (1, 5 and 30 mg/kg) also previously validated in our laboratory (19). A dose-response evaluation of colchicine was tested in three different concentrations: 100, 500 and 1,000  $\mu$ g/kg, with the 500 and 1,000  $\mu$ g/kg doses resulting in toxicity showing a 100% mortality rate.

### *Echocardiography*

Transthoracic echocardiography was performed prior to sacrifice at day 7 under general anesthesia with pentobarbital (50-70 mg/kg) using the Vevo770 imaging system (VisualSonics Inc., Toronto, Ontario, Canada) equipped with a 30-MHz probe. Left ventricle was visualized in the parasternal short axis view at the mid-ventricular level in the 2D-mode echocardiography. The image was optimized for the anterior wall and was zoomed to visualize the anterior pericardial structures. After optimization of the image, a B-mode and M-mode echocardiogram was

acquired for optimal spatial-temporal resolution and for measurements of the pericardial effusion, defined as the echo-free space between the two layers of the pericardium. We, however, considered images acquired through B-mode as they were found to be clearer in all subsets of animals.

Two investigators blinded to group allocation measured maximal pericardial effusion, expressed as the average among three different assessments.

#### *Assessment of pericardial thickness*

Hearts were collected and fixed in 10% formalin for 48 hours, dehydrated and embedded in paraffin. Five- $\mu$ m thick transverse sections were deparaffinized, re-hydrated and stained with standard hematoxylin and eosin protocol (Sigma Aldrich, St. Louis, MO, USA). Two investigators blinded to group allocation measured the thickness of the visceral pericardial layers with the aid of a computer morphometry analysis software (Image Pro Plus 6.0, Media Cybernetics, Silver Spring, MD, USA).

#### *Detection of the NLRP3 inflammasome components in pericardial specimens*

##### *Immunofluorescence staining*

ASC represents the scaffold of the NLRP3 inflammasome. Increased staining for ASC together with the formation of dense areas of aggregation provide evidence for the oligomerization of the inflammasome macromolecular structure (23,24).

In human pericardial samples, ASC expression was evaluated on 3- $\mu$ m transverse- sectioned hearts using an immunofluorescence technique, as previously described (25,26). Antigen epitopes were retrieved with 5 mM citrate buffer pH 6.0 at 95 °C. Slides were incubated overnight at 4°C with a primary antibody against human ASC (1:250; Sigma Aldrich, St. Louis, MO, USA). Anti-rabbit Alexa Fluor 594-conjugated (1:100; Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody for 4 hours at room temperature.

Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:100) for 5 min. Finally, slides were covered after adding SlowFade Antifade (Invitrogen, Carlsbad, CA, USA). The images (400 $\times$  magnification) were acquired through Olympus IX70 microscope and analyzed by cellSens software (Olympus Life Science, Waltham, MA, USA).

For murine samples, ASC, IL-1 $\alpha$ , and IL-1 $\beta$  expression were evaluated on 5- $\mu$ m transversal sections using a primary antibody against murine ASC (1:100; Sigma Aldrich, St. Louis, MO, USA), IL- $\alpha$  (1:100; R&D Systems, Minneapolis, MN, USA), and IL1- $\beta$  (1:25; Sigma Aldrich, St. Louis, MO, USA), respectively. Anti-rabbit Alexa Fluor 594-conjugated (1:100; Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody for 4 hours at room temperature. Additionally, heart slides were incubated with a primary antibody against cardiac  $\alpha$ -actin (1:200; Sigma-Aldrich, St. Louis, MO, USA) revealed by a secondary antibody anti-mouse Alexa Fluor 488-conjugated (1:100; Invitrogen, Carlsbad, CA, USA) to identify the myocardial cell and easily identify the pericardium, not positive for the cardiac  $\alpha$ -actin.

Quantifications were accomplished for all samples after consensus by two different investigators blinded to treatment allocation using a semi-quantitative scale ranging from 0 to 3 adapted from Abbate *et al.* (27) (**Table 1**).

#### Immunohistochemistry staining

Staining for NLRP3 and cleaved caspase-1 was performed using immunohistochemistry. After antigen retrieval, endogenous peroxidases were inactivated by a solution of 3% hydrogen peroxide for 15 min. A primary antibody raised against mature, active caspase-1 (cleaved Asp210, 1:50; ThermoFisher Scientific, Waltham, MA, USA) and NLRP3 (1:100; Novus Biologicals, Centennial, CO, USA) were incubated overnight at 4 °C. An anti-rabbit IgG conjugated to the horseradish peroxidase (HRP) secondary antibody was incubated for 2 hours at room temperature, followed by incubation with VECTOR® NovaRED™ Peroxidase Substrate (1:100; Vector Laboratories, Burlingame, CA, USA) and counterstaining with hematoxylin. Finally, slides were dehydrated and coverslipped with Cytoseal XYL (ThermoFisher, Waltham, MA, USA). Images were acquired using a Zeiss Axio Imager A.1 microscope (Zeiss, Oberkochen, Germany) using 10× objective for immunohistochemistry. Quantification was performed as previously mentioned (**Table 1**).

#### Real-time polymerase chain reaction

RNA was extracted from the parietal pericardium through affinity columns (ReliaPrep™ RNA Miniprep Systems, Promega corporation,

Madison, WI, USA) and converted to complementary DNA (cDNA) using the iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA, USA). Real-time polymerase chain reaction (PCR) was done using SsoAdvanced Universal SYBR Green Supermix (Biorad, Hercules, CA, USA), with a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Gene expression was carried for the following mRNAs: *Il-1 $\alpha$* , *Il-1 $\beta$* , and *Nlrp3*. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as a reference gene.

#### *Statistical analysis*

All data are expressed as mean and standard error of the mean (SEM). Analysis of variance (ANOVA) was used to analyze differences across treatment groups, followed by Student's t-test for multiple pairwise comparisons. Discrete variables between groups were evaluated using the Chi square ( $\chi^2$ ) test. Pearson's correlation coefficients were calculated to evaluate the correlation between pericardial effusion and pericardial thickness. A two-sided p-value <0.050 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 25.0 (IBM CO., Armonk, NY) and GraphPad Prism, version 8.2 for Windows (GraphPad Software, La Jolla, CA, USA, [www.graphpad.com](http://www.graphpad.com)).



## **RESULTS**

### *The NLRP3 inflammasome in human pericarditis*

The expression of the NLRP3 inflammasome components was detected in all pericardial samples of patients with chronic pericarditis experiencing an acute flare. In particular, NLRP3 and caspase-1 expression was found upregulated in the human pericardial samples (**Figure 2A-B**), with a significant increased expression compared with control subjects (NLRP3:  $1.9 \pm 0.15$  vs.  $1.21 \pm 0.1$ ,  $p=0.009$  and caspase-1:  $2.5 \pm 0.2$  vs  $1.4 \pm 0.09$ ,  $p<0.001$ , respectively). As read-out of inflammasome activation, ASC aggregates were measured by immunofluorescence staining. ASC was found significantly increased in pericarditis cases vs. controls (**Figure 2C**):  $2.4 \pm 0.2$  vs.  $1.1 \pm 0.3$  ( $p=0.006$ ). Hence, patients with chronic pericarditis presented an intensification of the NLRP3 inflammasome formation and activation compared to controls.

### *Induction of acute pericarditis by zymosan A in the mouse*

Based on literature, zymosan A was shown to induce NLRP3 inflammasome activation (12-15), and here we used it to develop a mouse model of acute pericarditis. In order to assess induction of pericarditis, we evaluated three parameters reflecting inflammation of the pericardium: presence of pericardial effusion, pericardial thickness, and ASC expression. Seven days after the intrapericardial injection of zymosan A, mice developed a significantly larger pericardial effusion (+83%) compared to sham ( $p<0.001$ ) (**Figure 3A and Figure 4C-D**). This finding was found already after 3 days from surgery, with zymosan A-

treated mice showing a larger effusion compared with sham mice ( $p < 0.001$  in B-mode and  $p = 0.004$  in M-mode; **Figure 4A-B**). A morphometrical analysis on hematoxylin and eosin stained heart sections was carried out at day 7 and revealed a significant increase (45%) in the visceral pericardial thickness compared to sham-operated mice ( $p = 0.016$ ; **Figure 3B**). No fibrinous deposits were observed at a macroscopic or microscopic evaluation at the time of harvesting. Positive correlations between pericardial effusion (assessed through echocardiography) and pericardial thickness (assessed through histology) were found (**Figure 5**). At day 7, mice treated with zymosan A showed a 60-fold increase expression of ASC compared to sham mice indicating activation of the NLRP3 inflammasome ( $p < 0.001$ ; **Figure 3C**).

Recent findings highlighted the possible role of both IL- $\alpha$  and IL1- $\beta$  in driving the clinical symptoms (11,28). For this reason, we investigated the presence of these two cytokines in our animal model of acute pericarditis. A subset of mice, including a group were injected with zymosan A and one group with sham mice, were sacrificed 3 days after surgery to perform molecular biological analysis and immunofluorescence for IL- $\alpha$  and IL1- $\beta$ . A greater expression of IL- $\alpha$  and IL1- $\beta$  was found both at a transcriptional (**Figure 6A**) and translational level (**Figure 6B-C**) in mice treated with zymosan A compared with sham-operated mice.

Cardiac function was preserved in mice injected with zymosan A and in sham mice both at 3 and 7 days.

In sum, the mouse model of acute pericarditis replicates several common features mediated by the local inflammation of the pericardium as observed in patients with pericarditis.

#### *Effects of ibuprofen in experimental pericarditis*

Ibuprofen, a cyclooxygenase-2 (COX-2) inhibitor, is currently used as a first-line therapy along with colchicine in patients with acute pericarditis (1). Treatment with ibuprofen in mice with acute pericarditis reduced pericardial effusion compared to vehicle-treated mice by 42% ( $p<0.001$ ) (**Figure 7A**). In contrast, ibuprofen failed to prevent pericardial thickening (**Figure 7B**) and ASC expression (**Figure 7C**).

#### *Effects of NLRP3 inflammasome inhibition in experimental pericarditis*

In order to block the NLRP3 inflammasome activity, we used colchicine, recently recognized as an NLRP3 inhibitor and largely used both in acute and recurrent pericarditis (10) as well as the NLRP3inh (16673-34-0), generated here at Virginia Commonwealth University (16) and previously tested by our group in experimental myocardial ischemia (17,18). Colchicine and the NLRP3inh 16673-34-0 significantly reduced pericardial effusion at day 7 by 28% and 46%, respectively ( $p<0.010$  for both; **Figure 8A**). Colchicine was ineffective in reducing pericardial thickness, whereas the NLRP3inh 16673-34-0 significantly reduced the pericardial thickening by 32% ( $p=0.003$ ; **Figure 8B**). ASC aggregation was reduced at day 7 both by colchicine (-93% vs. vehicle-treated mice,  $p<0.001$ ) and the NLRP3inh (-78% vs. vehicle-treated mice,  $p<0.001$ ; **Figure 8C**).

### *Effects of IL-1 blockade in experimental pericarditis*

IL-1 blockade was evaluated using two different drugs: anakinra, an IL-1 receptor antagonist, proposed as treatment for recurrent pericarditis, and IL-1 trap, a cytokine trap that prevents soluble IL-1 $\alpha$  and IL-1 $\beta$  from binding to their receptor, IL-1R1. Treatment with anakinra twice daily attenuated pericardial effusion by 13% compared to the vehicle group ( $p < 0.050$ ; **Figure 9A**). Similarly, IL-1 trap given every 48h reduced pericardial effusion by 43%, 35% and 33% at all 3 doses tested, respectively, compared with the vehicle-treated group ( $p < 0.010$  for all comparison; **Figure 9D**). Anakinra reduced pericardial thickening by 20% ( $p < 0.050$  vs. vehicle; **Figure 9B**). Similarly, the IL-1 trap consistently reduced pericardial thickening (-36%, -42%, and -44%, respectively,  $p < 0.001$  for all; **Figure 9E**). Finally, the formation of the inflammasome, measured as ASC aggregates, was significantly reduced by anakinra (-75% vs. vehicle,  $p < 0.001$ ; **Figure 9C**) and by all of the three doses of IL-1 trap (-85% for 1 mg/kg, -69% for 5mg/kg and -96% for 30mg/kg,  $p < 0.001$  for all; **Figure 9F**).

**Table 2** summarizes main findings of the different tested drugs with regard to the efficacy in reducing the pericardial effusion, pericardial thickness, and ASC expression.

## **DISCUSSION**

In the present study we were able to describe for the first time the presence of NLRP3 inflammasome expression in human pericardial specimens from patients with pericarditis. We also demonstrated that the phenotype of acute pericarditis – thickening of the pericardium, presence of an effusion, and inflammation associated with NLRP3 expression – can be reproduced in a mouse model with the instillation of zymosan A within the pericardial space. The expression of IL-1 $\alpha$  and IL-1 $\beta$  in the pericardium of mice with acute pericarditis was also demonstrated with a higher expression compared with sham mice. Finally, different treatments for pericarditis have been tested and results are summarized in **Table 2**.

Zymosan A is a TLR-2 agonist and can induce NLRP3 inflammasome pathway activation (12,15,17,29). Our mouse model showed that the NLRP3 inflammasome promotes the inflammatory changes occurring in acute pericarditis, i.e. pericardial effusion and pericardial thickening. As supportive evidence, drugs known to inhibit the NLRP3 inflammasome pathway – i.e. colchicine, NLRP3inh 16673-34-0, anakinra, and IL-1 trap – were found to reduce all or most of the above-mentioned inflammatory effects.

Other models of pericarditis were proposed in the past, using irritating agents capable to activate the NLRP3 inflammasome, although there was no knowledge about it at the time of publication. Pagé *et al.* used hydrated magnesium silicate (talc) dusted on the pericardium of dogs (30). Now we know that silicate crystals can induce the NLRP3 inflammasome activation (24). The intrapericardial injection of heat-killed staphylococci and Freund's adjuvant in sheep, as performed by Leak *et*

*al.*, showed the different stages of the pericardial inflammation, allowing to evaluate the inflammatory cell infiltration, the production of fibrin, the formation of pericardial adhesions until the final resolution with fibrosis (31). The bacterial products of the Freund's adjuvant (containing aluminum) are canonical stimuli for the NLRP3 inflammasome activation (32), resulting again in an inflammasome-dependent model of acute pericarditis. Recently, a mouse model mimicking pericardial inflammation was performed with the intrapericardial injection of blood, minocycline, picibanil, and talc through a single-shot injection (33). Compared to others, our model has obvious advantages. In fact, the use of large animals, such as sheep or dogs, can better mimic what happens in humans, but also has clear disadvantages, primarily related to the complexity and the costs associated with their housing and handling. We therefore believe that the reproduction of the pathophysiological alterations of acute pericarditis in the mouse may represent an easy and widely replicable model of pericarditis. The model of acute pericarditis reflecting the modifications of the acute pericardial inflammation may, however, not be adequate to evaluate long-term effects of pericardial inflammation, as occurs in chronic pericarditis. However, we are interested in tackling the acute phase of the disease presenting with disabling symptoms, such as chest pain, and therapies should be targeted to block the detrimental effects of the acute phase of the disease in order to prevent recurrences (3). Moreover, the use of genetically modified mice offers the advantage to study new pathways involved in the pathophysiology of pericarditis.

Although generally self-limiting, pericarditis can be refractory to standard therapies and/or recur in approximately 30% of cases (34,35). In spite of this, its pathophysiology is still poorly understood and current therapeutic strategies are limited and not always effective in avoiding recurrences. Indeed, no drug is approved by the US FDA for the treatment of acute pericarditis. Currently, NSAIDs are commonly used as a first-line treatment but, despite being generally effective in controlling symptoms, they did not show a change in the disease course and are associated with a significant recurrence rate after discontinuation of treatment (1). Accordingly, we found that ibuprofen treatment affects some of the features of acute pericarditis in a mouse model. Indeed, ibuprofen reduced pericardial effusion but did not prevent the formation of the inflammasome nor the pericardial thickening. This effect was somewhat expected given its downstream effect on COX-2 with respect to the NLRP3 inflammasome (4).

The limited therapeutic armamentarium is in part due to the scarcity of animal models replicating the pathophysiological modifications occurring in acute pericarditis. With this regard, our mouse model of pericarditis with zymosan A mimics the acute injury occurring into the pericardium through which the NLRP3 inflammasome can be activated. Indeed, the NLRP3 inflammasome activation was only indirectly hypothesized until now, but not yet demonstrated. In fact, the positive results with anakinra coming from the AIRTRIP (Anakinra—Treatment of Recurrent Idiopathic Pericarditis) trial (11) and others (36) strongly supported the hypothesis that the NLRP3 inflammasome importantly contribute to the clinical symptoms of the pericardial syndrome. In

addition, we now demonstrated that the NLRP3 inflammasome is expressed by pericardial cells and activates during an acute flare of pericarditis leading to the release of IL-1 $\alpha$  and IL-1 $\beta$ , that explain the typical inflammatory symptoms complained by patients. Release of IL-1 $\alpha$  from dying cells may also trigger an inflammatory response in pericarditis (6,8). While IL-1 has been extensively studied in cardiovascular (i.e. acute myocardial infarction and heart failure (37-39)) and rheumatic diseases (i.e. rheumatoid arthritis, gout, and adult onset Still's disease (40)), very little is known about its role in pericarditis (3). In this manuscript, we reported for the first time the presence and activation of the NLRP3 inflammasome in human samples of chronic pericarditis. This finding was corroborated by the concurrent presence of all of the three components of the inflammasome at the same time (ASC, NLRP3, and caspase-1) (6,23,24). We were also able to demonstrate an increased expression of IL-1 $\alpha$  and IL-1 $\beta$  in the pericardium of mice treated with zymosan A compared with sham mice. This suggests that both these cytokines are strongly involved in the pathogenesis of acute pericarditis, as suggested by a recent report in which canakinumab failed to improve steroid-dependent idiopathic recurrent pericarditis in two children (28). In addition, blocking IL-1 using both anakinra and IL-1 trap in mice with acute pericarditis was highly effective in decreasing pericardial effusion and thickness as well as blunting the NLRP3 inflammatory response. Anakinra required a high and frequent dosing, consistent with its mechanism of action as a receptor antagonist, whereas the IL-1 trap was effective across a wide range of dosing and less frequent administrations, functioning as an entrapment for circulating IL-1 $\beta$  and IL-1 $\alpha$ . In this



regard, the design of RHAPSODY, a double-blind, placebo-controlled, randomized-withdrawal phase 3 trial (NCT03737110) testing rilonacept in patients with multiple episodes of recurrent pericarditis has been published (41).

Colchicine is now considered among first-line treatments for both acute and recurrent pericarditis based on different clinical trials showing its efficacy (1). The main mechanism of colchicine is not completely elucidated. Colchicine relies on the inhibition of microtubule polymerization and it prevents neutrophil migration (42). Colchicine has been shown to interfere with the formation of the macromolecular structure of the NLRP3 inflammasome and preventing the association of its components and IL-1 $\beta$  release (10,43,44). In our study, colchicine, at the dose tested, significantly reduced the pericardial effusion and ASC aggregation in the zymosan A pericarditis model, while it failed to prevent the pericardial thickening. However, the effects of higher doses of colchicine could not be tested due to the toxicity of the drug. The use of the NLRP3inh was effective in preventing inflammasome formation, reducing pericardial effusion, and reducing pericardial thickening. This is of great interest because although colchicine is effective, a significant proportion of patients are refractory or cannot tolerate colchicine and require additional immunomodulating drugs. The role of the NLRP3inh is consistent with the clinical efficacy of IL-1 blockers in preliminary case reports (36,45-51).

This study is, however, not without limitations. Human samples of pericarditis came from patients with long-term, chronic pericarditis needing pericardiectomy and this may represent a selection bias.

Unfortunately, pericardiectomy is neither routinely indicated in acute pericarditis nor ethically feasible but reserved only for those cases presenting with a constrictive evolution. The available pericardial specimens were limited in number and additional samples may be needed to further confirm and expand our results. The small surgical procedures require specialized skills, a learning curve, and are time-consuming. The mouse model presents clear challenges in the assessment of symptoms and behavioral effects. Additionally, the surgery in the mouse demands the use of narcotics for pain control, which means that chest pain, a common symptom in acute pericarditis, cannot be adequately assessed. Pericarditis is often considered related to a viral infection or an immune response to the virus, and therefore this model may not recapitulate all the upstream events leading to the inflammatory response. This animal model was conceived to explore the pathophysiological events occurring in acute pericarditis, and not to explore long-term effects as in chronic pericarditis. Chronic intrapericardial injection of zymosan A is not feasible as it necessitates anesthesia and surgery at every time and should be repeated at least every 7 days, thus possibly jeopardizing long-term survival of mice. In addition, the persistent stress induced on the pericardium by the needle may create local inflammation, that could represent a confounder in the analysis of results. As the model was designed with a translational perspective in mind, we chose to use pharmacological inhibitors and chose not to study genetically modified (i.e. NLRP3, IL-1 or IL-1R knock-out) mice in this study. A systematic characterization of the cellular infiltrate in the pericardium was not performed at this time. A post-hoc

adjustment, such as Dunnett's test, was not considered for multiple pairwise comparisons therefore potentially increasing the risk of an inflated type I error. Despite its limitations, this model may represent a first step to explore novel pathophysiological mechanisms and therapeutics in the future.

## ***CONCLUSION***

We herein demonstrated for the first time the expression and the activation of the NLRP3 inflammasome both in humans and in mice with pericarditis. Additionally, the instillation of zymosan A within the murine pericardium reproduces, through an NLRP3 inflammasome-dependent mechanism, the phenotype of acute pericarditis, which is responsive to colchicine, NLRP3inh, anakinra, and IL-1 trap. Using a small rodent model brings many advantages, including limited cost, ease of use, and the ability to manipulate genetic expression. Through this option, it will be possible to plan the development of new therapeutic approaches targeting NLRP3-driven pathways for further mechanistic and translational studies. The study also shows powerful inhibition of the inflammatory response in this pericarditis model with inhibitors of IL-1.

## **TABLES AND FIGURES**

**Table 1. Semi-quantitative scale for histological quantification of the NLRP3 inflammasome.**

|   |                     |   |
|---|---------------------|---|
| 0 | No expression       | No visible aggregates   |
| 1 | Low expression      | Few aggregates (<1/high power field) or mild diffuse stain without aggregates   |
| 2 | Moderate expression | 1-5 aggregates/high power field or diffuse stain with few aggregates            |
| 3 | High expression     | Diffuse intense staining with many cytoplasmic aggregates (>5/high power field) |

**Table 2. Main effects of different pharmacological strategies in the animal model of acute pericarditis.**

| <b>Treatment</b> | <b>↓ Pericardial effusion</b> | <b>↓ Pericardial thickness</b> | <b>↓ ASC expression</b> |
|------------------|-------------------------------|--------------------------------|-------------------------|
| Ibuprofen        | <b>++</b>                     | <b>=</b>                       | <b>=</b>                |
| Colchicine       | <b>+</b>                      | <b>=</b>                       | <b>+++</b>              |
| NLRP3 inhibitor  | <b>++</b>                     | <b>++</b>                      | <b>+++</b>              |
| Anakinra         | <b>+</b>                      | <b>+</b>                       | <b>+++</b>              |
| IL-1 trap        | <b>++</b>                     | <b>++</b>                      | <b>+++</b>              |

The efficacy of the different treatments on reducing pericardial effusion, pericardial thickness, and ASC expression are showed in comparison with vehicle (mice injected with zymosan A only).

Data for IL-1 trap are provided as an average of the three tested doses.

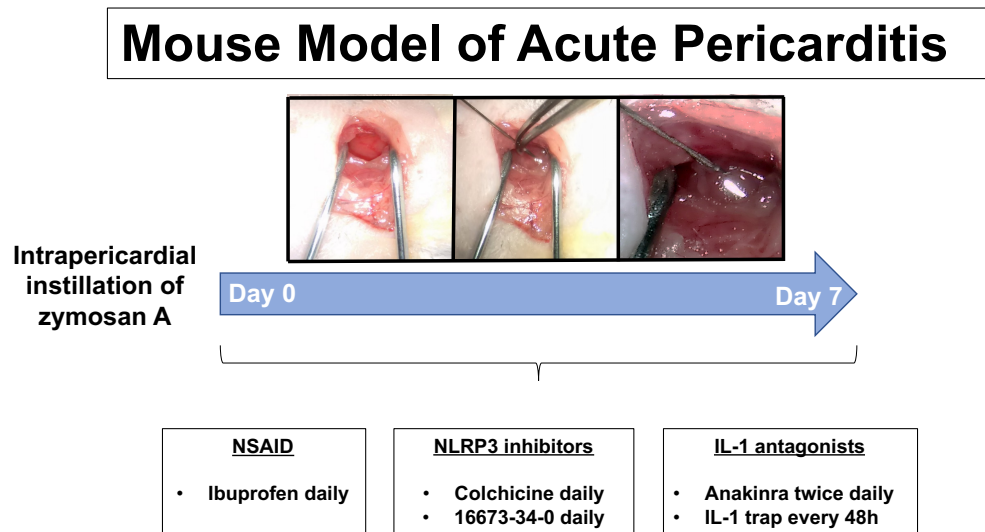
**=**: no change

**+**: reduction between 0 and 30%

**++**: reduction between 31% and 60%

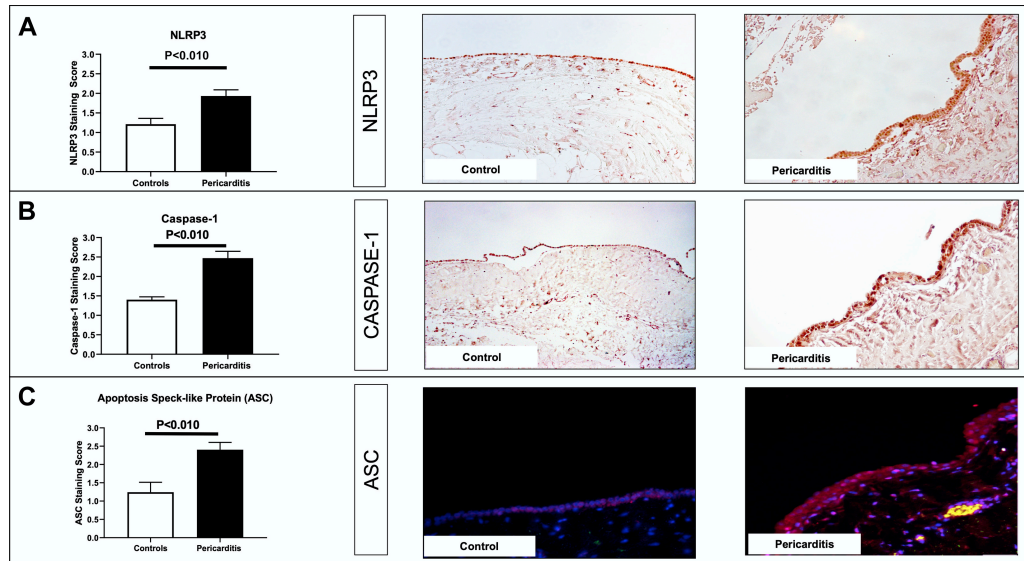
**+++**: reduction between 61% and 100%

# Figure 1



**Figure 1. Schematic representation of the experimental mouse model of acute pericarditis.** Under general anesthesia, 1 mg of zymosan A (in 50  $\mu$ l of sterile NaCl 0.9%) was injected in the pericardial sac of the mouse on day 0. Sham control procedures were performed injecting an equal volume of sterile NaCl 0.9% in the pericardial sac. Selected drugs were administered after the surgery was completed until day 7. On day 7, mice underwent echocardiography and were euthanized.

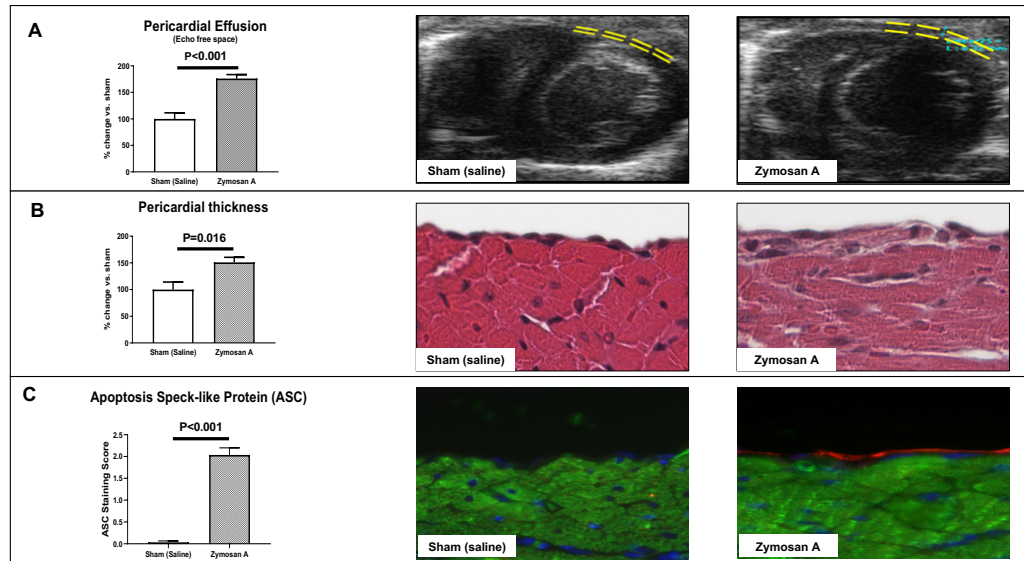
## Figure 2



**Figure 2. Upregulation of NLRP3 Inflammasome components in human pericardial specimens of patients affected by pericarditis. A)** Immunohistochemistry reveals upregulation of the NLRP3 protein following pericarditis in human samples (original magnification 40×). **B)** Caspase-1 upregulation detected by immunohistochemical staining (original magnification 40×). **C)** Immunofluorescence staining of ASC (in red) showing aggregates in the pericardium. Counterstaining of the nuclei achieved with DAPI (blue) (original magnification 40×).

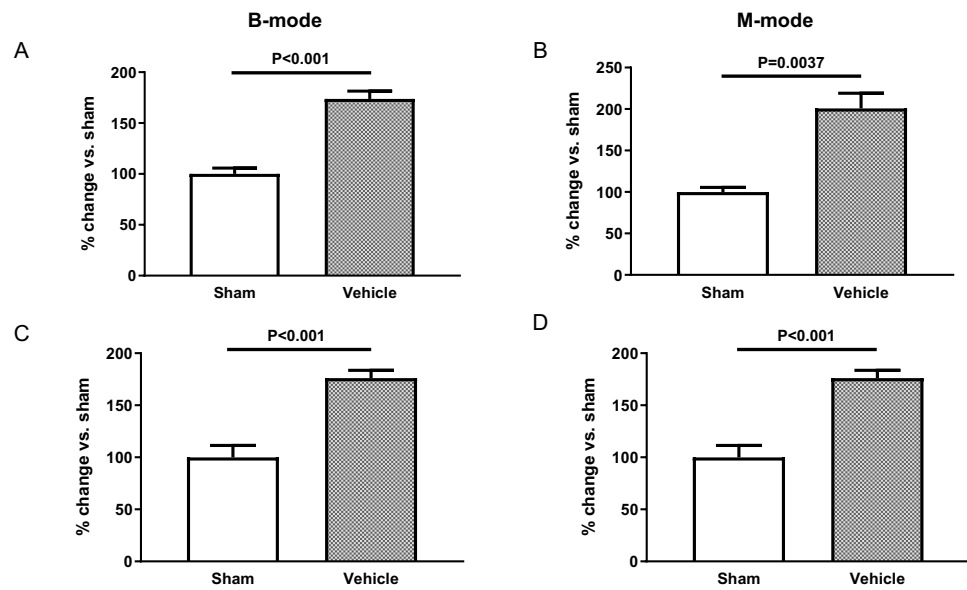


## Figure 3



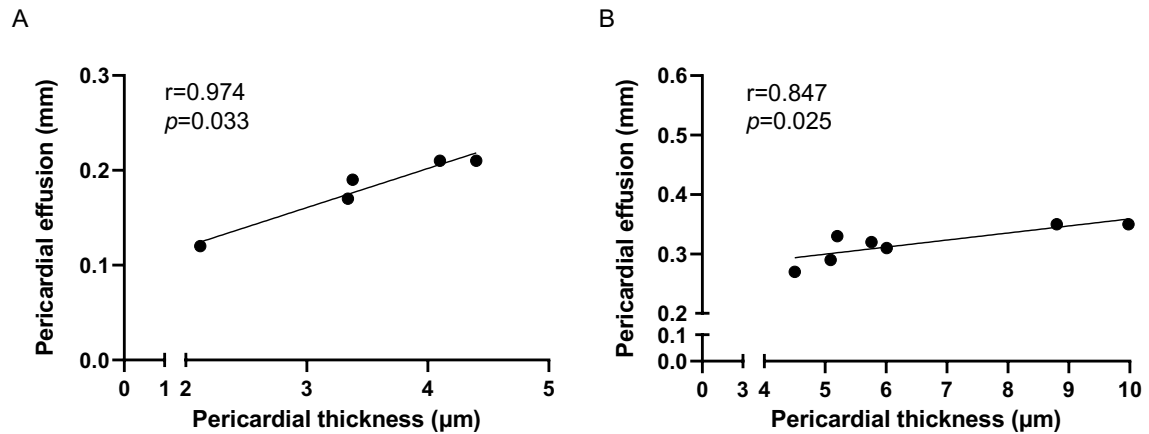
**Figure 3. Assessment of pericarditis in mice. A)** Pericardial effusion was measured at 7 days by transthoracic echocardiography as echo-free space between the two layers of the pericardium. Instillation of zymosan A produces a significant increase in pericardial fluid compared to sham controls injected with saline. **B)** Pericardial thickening was measured at day 7 by histological assessment of H&E stained heart slides. Zymosan A increased significantly the thickness of the visceral pericardial layer compared to sham (original magnification 40×) **C)** Immunofluorescence staining for ASC (in red) and cardiac  $\alpha$ -actin (green) in the pericardium of mice with experimental pericarditis. DAPI was used to counterstain the nuclei (blue) (original magnification 40×).

## Figure 4



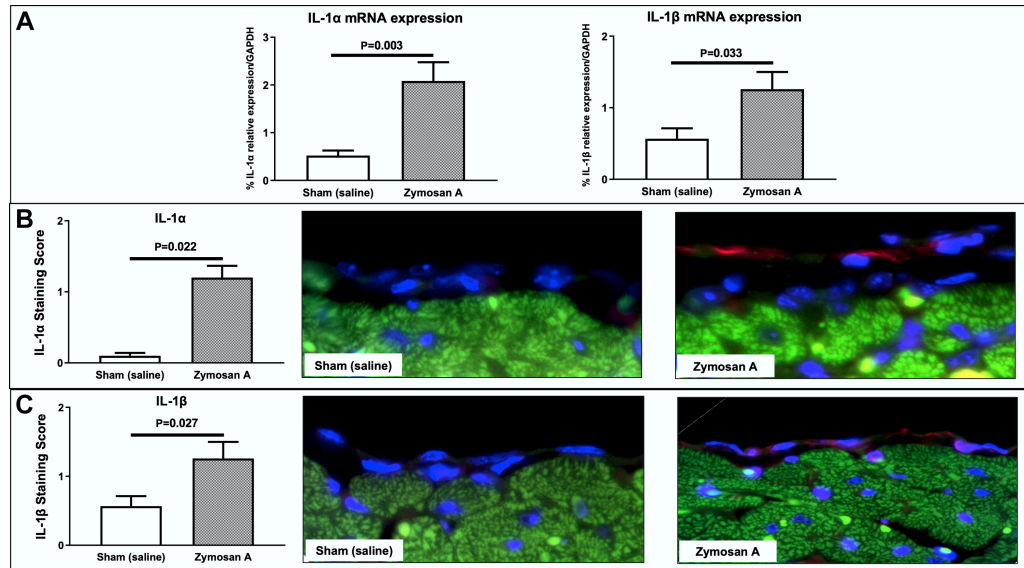
**Figure 4. Echocardiographic assessment of pericardial effusion at day 3 and 7.** Assessment of pericardial effusion was performed through echocardiography using both B-mode and M-mode modalities at 3 and 7 days (panel A-B and C-D, respectively). p for Student's t-test.

## Figure 5



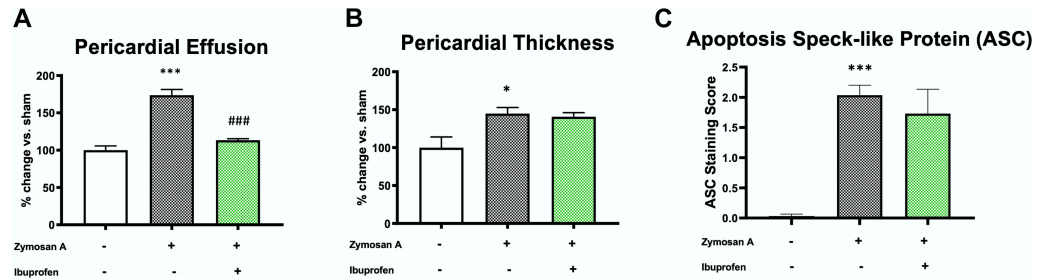
**Figure 5. Correlations between pericardial effusion and pericardial thickness at day 7.** Correlations between pericardial effusion (assessed through echocardiography) and pericardial thickness (assessed by histology) at day 7 before sacrifice are shown for sham- and vehicle-treated animals in panels A and B, respectively.  $p$  for Spearman's rank correlation.

## Figure 6



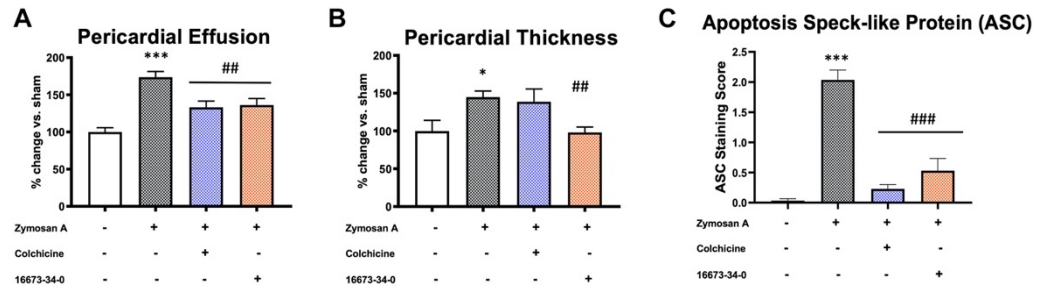
**Figure 6. Assessment of IL-1α and IL-1β in mice. A)** IL-1α and IL-1β mRNA relative expression in the parietal pericardium was measured at three days by real-time polymerase chain reaction (PCR). Mice treated with intrapericardial instillation of zymosan A showed a significant increase in mRNA expression of both IL-1α and IL-1β compared with sham controls injected with saline. **B)** Pericardial expression of IL-1α was measured at day 3 by immunofluorescence staining. Zymosan A-treated mice had a higher expression of IL-1α compared with sham. IL-1α positive cells are stained in red, while DAPI was used to counterstain the nuclei (blue). The myocardium appears green by natural autofluorescence. Original magnification 40×. **C)** Immunofluorescence staining for IL-1β (in red) in the pericardium of mice with experimental pericarditis demonstrated a higher expression of this cytokine in zymosan A-treated mice compared with sham controls. IL-1β is stained in red, while DAPI was used to counterstain the nuclei (blue). The myocardium appears green by natural autofluorescence. Original magnification 40×.

## Figure 7



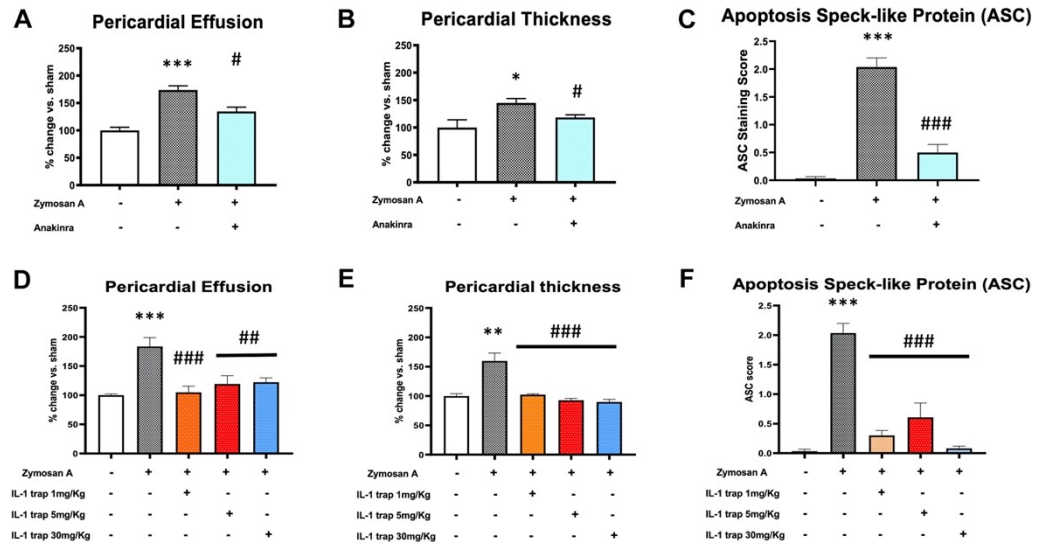
**Figure 7. Effects of ibuprofen treatment in the experimental model of pericarditis in mice.** **A)** Pericardial effusion was measured at 7 days following daily treatment with ibuprofen (100 mg/kg). Daily injection with ibuprofen significantly reduced fluid accumulation. **B)** Treatment with ibuprofen did not reduce pericardial thickness. **C)** ASC aggregates were not reduced by treatment with ibuprofen. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs sham-treated mice; ### $p < 0.001$  vs vehicle-treated mice.

## Figure 8



**Figure 8. Inhibition of the NLRP3 inflammasome in mice with acute pericarditis.** **A)** Pericardial effusion measured at 7 days following daily treatment with colchicine (100  $\mu$ g/kg) and NLRP3inhibitor 16673-34-0 (100 mg/kg). Colchicine and 16673-34-0 significantly reduced pericardial effusion in mice with pericarditis. **B)** Treatment with the NLRP3 inhibitor 16673-34-0, but not colchicine, showed a significant reduction of the pericardial thickness following acute pericarditis in mice compared to the vehicle-treated group. **C)** ASC aggregates in the pericardium of mice with acute pericarditis were significantly reduced by colchicine and 16673-34-0. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs sham-treated mice; ## $p < 0.01$ , ### $p < 0.001$  vs vehicle-treated mice.

# Figure 9



**Figure 9. IL-1 blockade in experimental acute pericarditis.** Pericardial effusion, thickness, and formation of ASC aggregates were measured 7 days after bi-daily treatment with anakinra (100 mg/kg) and IL-1 trap. Anakinra significantly reduced **A)** pericardial fluid accumulation **B)** thickening of the pericardium and **C)** ASC aggregation following acute pericarditis in mice.

A dose response of IL-1 trap was tested in mice subjected to experimental pericarditis. Treatment with IL-1 trap every 48h significantly reduced **D)** pericardial effusion **E)** pericardial thickness and **F)** inflammasome formation with each dose tested. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs sham-treated mice; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs vehicle-treated mice.

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